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A cryopreservative procedure for storing cultivated and uncultivated amniotic fluid cells in liquid nitrogen

S PENTZ AND H HÖRLER

From the Abteilung Klinische Genetik, University of Ulm, D-7900 Ulm, Federal Republic of Germany

SUMMARY The cryopreservation of cultured and uncultured amniotic fluid cells is of speci importance in prenatal diagnosis. The freezing procedure presented gave a cell recovery of abo 95% for cultivated human amniotic fluid cells combined with a rapid appearance of mitosis aft inoculation.

A number of inborn errors of metabolism can be detected prenatally in amniotic fluid cells. Diagnosis of these metabolic defects requires cultivated control cells from the amniotic fluid. For this reason, it is important to be able to store cells for long periods of time for prenatal diagnosis. However, variation in metabolic processes often takes place during the cultivation, depending on the cell culture conditions, the number of passages, and the momentary phase of growth. Heukels-Dully and Niermeijer[1] estimated a time-related variation in lysosomal enzyme specific activity in subcultivated amniotic fluid cells, but it should also be noted that activity of some lysosomal enzymes are relatively stable in primary cultures. It is for this reason that adequate control cultures for biochemical analysis should be available at all times, hence the need for uncultured amniotic fluid cells stored in liquid nitrogen.[2]

Together with glycerol, dimethyl sulphoxide (DMSO) is the most useful cryopreservative agent. The latter has a high penetration rate through cell membranes and can be easily washed out after the thawing process.[3 4] In addition, DMSO possesses good colligative properties.[5] Many investigators have shown that DMSO is especially toxic when the temperature is increased (0 to 37°C).[6–8] Hak *et al*[9] and Alink *et al*[10] showed that the toxic effects of DMSO on beating heart cells in vitro could be reduced when DMSO was added slowly to cells before the freezing process and its concentration slowly reduced on thawing. It is assumed that by this procedure the damaging effect of DMSO on the cell membrane by osmotic stress can be avoided.

The following report is a description of a cell freezing process which does not subject the cells t harsh treatment, has a high cell recovery, and a rap cell regeneration after thawing.

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Materials and methods

FREEZING PROCEDURE

Uncultivated amniotic fluid cells (5 ml amniot fluid/sample) were centrifuged for 10 minutes a 135 *g* and the amniotic fluid discarded. Cultivate amniotic cells were washed with Hank's balance salt solution (BSS) and treated with 0·25% trypsi and 0·2% EDTA (6:4) to release adherent cell Serum was added to the resulting cell suspension i a ratio of 2:1 and centrifuged in polycarbonate tub (Greiner, D-7440 Nürtingen), resuspended, an centrifuged again. The supernatant was discarde and the cells resuspended in Eagle's MEM mediu containing Earle's salts, 20% FCS (Seromec D-8000 München), 5% L-glutamine, 100 I penicillin/ml, and 100 µg streptomycin/ml. Th tubes were then placed in iced water. For a fina DMSO concentration of 5%, a 9·2% DMS(solution in MEM was prepared and 0·6 ml of th precooled solution was added drop by drop to 0·5 m of the cell suspension. Addition of this solution t the cell suspension was carried out within 6 minute: It was performed in our laboratory using a automatic time-pipette.

One ml of the DMSO-cell suspension was adde aseptically to precooled freezing vials (Polypropylen Greiner) and closed tightly. The optimal cell cor centration of amniotic fluid cells was found to b between 5×10^5 and 2×10^6 cells/ml. For freezing a liquid nitrogen container type LR-33-10 (Unio

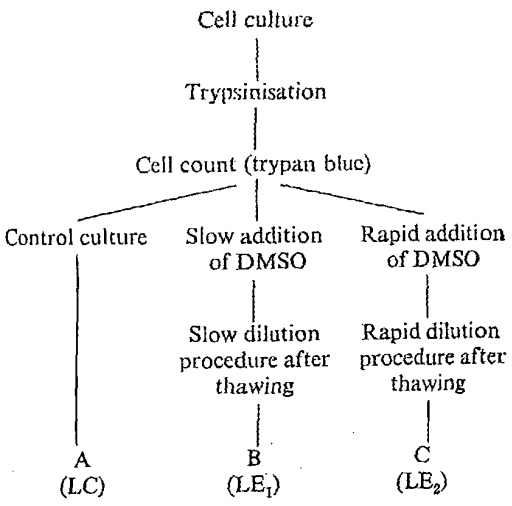
Carbide) was used from which the normal lid was removed. In its place was inserted a special adaptor lid together with a freezing vial holder (BF-6). The freezing vial holder was set in the E position and filled with 5 vials. The rate of freezing was measured as approximately 1·6°C/min in the liquid phase and 1·4°C/min in the solid phase, as recommended by the manufacturers. Freezing to −70°C then takes about 100 minutes. This time is constant when the freezing container is 70 to 80% full of liquid nitrogen.

THAWING OF CELLS

The freezing vials were taken out of the liquid nitrogen container and put directly into a 37°C water bath. The vials were shaken in the water bath until only a small ball of ice remained. The cell suspension was then pipetted into a centrifuge tube which had previously been cooled, and 9 ml of cooled culture medium was added in drops within 9 minutes. Since the cells adhere to glass, plastic tubes were used. The cells were washed twice with medium and centrifuged between each wash. The cells were then resuspended in a culture flask with medium supplemented with 20% FCS.

EXPERIMENTAL METHOD

Cells from a culture flask were divided into three equal parts and the recovery determined according to the following scheme.



Twenty-four hours after cell cultivation, the floating cells were counted and dead cells were evaluated using the trypan blue method (LC = dead cells in the control culture; LE = dead cells after cryopreservative procedure).

Calculation of the recovery =

$$\frac{100 - (\text{LE/ml} - \text{LC/ml}) \times 100}{\text{total plated cell count/ml}}$$

Results

The results from cell cultures show that recovery is significantly higher ($p<0{\cdot}01$) when DMSO is added slowly before freezing and slowly diluted after thawing than when DMSO is added rapidly. The latter process also gives rise to a greater variation in results (table 1). Furthermore, as with normal cultures, cell division of cultured amniotic cells subjected to cryopreservation by the slower procedure

TABLE 1 *Recovery of cultivated amniotic fluid cells after slow (B) and rapid (C) DMSO addition*

Culture No	*Total cell count*	*Recovery*	
		B (%)	*C* (%)
A 884	1 045 000	94·74	83·68
A 969	1 975 000	91·04	78·20
A 1230	850 000	91·18	82·65
A 1038	1 270 000	97·24	82·48
Ac 118	755 000	97·15	84·83
A 348	1 380 000	90·29	62·39
A 326	2 270 000	100·57	90·42
A 856	1 530 000	96·86	77·35
Ac 98	960 000	94·79	82·92
A 1213	880 000	89·77	63·24
A 1615	625 000	95·09	84·97
A 1469	1 300 000	91·15	89·73
A 828	920 000	91·85	75·33
A 924	1 485 000	94·48	84·88
A 873	1 150 000	98·00	85·65
A 1484	970 000	99·43	84·07
		$\bar{x} = 94{\cdot}60 \pm 3{\cdot}42$	$80{\cdot}36 \pm 7{\cdot}71$

TABLE 2 *Number of colonies from uncultured amniotic cells inoculated before and after the freezing procedure*

Culture No	*Gestation week*	*Colonies from cells of 5 ml amniotic fluid*	
		Direct culture	*Culture after freezing*
Ac 50	15	8	7
Ac 51	14	5	12
Ac 52	14	14	11
Ac 53	14	7	9
Ac 54	18	9	4
Ac 56	17	2	5
Ac 57	18	17	18
Ac 58	14	10	8
Ac 59	14	7	7
Ac 61	14	8	10
Ac 62	17	22	19
Ac 63	16	12	11

occurs between 2 and 6 hours after inoculation (B, LE_1), while division of the cells using the rapid procedure occurs between 10 and 12 hours. Table 2 shows that there is no difference between the number of colonies from uncultured amniotic cells inoculated before and after the freezing procedure.

Discussion

The results indicate that the modified cryopreservation procedure described here ensures a rapid supply of uncultivated and cultivated amniotic cells. In addition, no difference was observed between the number of colonies of uncultured amniotic fluid cells before or after storage in liquid nitrogen. With respect to the appearance of the first dividing cells, we did not find any difference between direct culture and cultivation after freezing and thawing.[2] A recovery of approximately 95% and an earlier appearance of mitotic cells were found in cultures of cultivated amniotic cells after the special slow freezing and thawing method. Using the rapid procedure a lower recovery was obtained (80%) and mitosis of the cells occurred at a later stage than by the slower method. Both procedures may be compared favourably with those of some other investigators which may possibly be the result of the careful handling of cells before cryopreservation, for example, through the rapid inactivation of the enzymatic activity of trypsin after detachment of the cells from the cultivation surface. Ashwood *et al*[11] obtained a similarly high recovery of 91% in cultures of Chinese hamster ovary fibroblasts after slow addition of DMSO to the cell suspension 10 to 15 minutes before the freezing procedure was started. The low concentration of DMSO (5%), together with the low but increasing temperature (4°C to 16°C), obviously reduces the toxicity of the cryopreservative agent. Niermeijer *et al*[2] showed that a DMSO concentration of 4% was just within the protection range for uncultivated amniotic fluid cells. An even lower DMSO concentration has, according to Alink *et al*,[10] extremely reduced cryopreservative properties, as well as increasing the probability of micro-damage to the cells.[12] Many authors have described microscopic damage to cell membranes and organelles of living cells after suboptimal freezing procedures.[13–15] Alink *et al*[16] showed, using electron microscopy, that only after an 8-day period is regeneration of microscopic damage complete.

With regard to cell recovery, a comparison in procedure with other laboratories is difficult since many methods have been used in the past. Recovery is, however, frequently measured as the 'plating efficiency' which has been defined by Fedoroff[17] as the "percentage of inoculated cells which gave rise to colonies". So that an optimal number of colonies can be counted, a correspondingly low cell dilution must be inoculated. However, plating a low cell concentration of mammalian cells usually results in little or no culture growth. For this reason, other methods have been described to measure the recovery of diploid mammalian cells.[15 18 19] The method employed here to determine the cell recovery for cultivated amniotic cells is a combination of the 'floating cell method' described by Priest *et al*[18] and the method described by Hunt *et al*,[15] in which comparison is made with unfrozen control cultures, both methods relying on a measurement after a 24 hour cell incubation period. A trypan blue determination of dead cells between 2 and 8 days of incubation would indicate lethal damage, for example, after a suboptimal cryopreservation process.[10] However, the results could be questioned on the grounds of uncontrollable external toxic factors.[19] Counting floating cells after incubation[18] is therefore considered by some authors to be a better choice for determining cell recovery than counting and staining after cell trypsinisation, since an adequate control is not possible in the latter case. A comparison with an unfrozen control culture is similarly necessary since cellular damage can occur, for example, through trypsinisation[16] followed by cultivation, and cannot be separated from toxic substances or processes which occur in the cryopreservation system.

It is for this reason that further experiments in this field will be concerned with cell lines from clones morphologically different cells obtained from amniotic cell cultures and the recovery of these cells measured after cryopreservation using different conditions.

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Requests for reprints to Dr S Pentz, Abteilung Klinische Genetik, Universität Ulm OE, D-7900 Ulm, Federal Republic of Germany.